# EXHIBIT 6d

As indicated above, the chemical structures of the APAP metabolite AM404 are built on the same backbone as the endocannabinoid, anandamide. While Δ9THC lacks this backbone, it does bind with the same receptors as anandamide, including CB1.[168] Rodent animal studies report that even low doses of cannabinoid compounds can result in atypical locomotor activity, cognitive impairments, altered emotional behavior and clinical studies support this interaction.[169] There were similar findings in children with prenatal *in utero* exposure to cannabis (Δ9THC) that have prompted public health announcements (Figure 16). Reported neurobehavioral abnormalities include increased hyperactivity, impulsivity, and inattention symptoms,[170] First trimester exposure also predicted deficits in Wide Range Achievement Test-Revised (WRAT-R) reading and spelling scores and a lower rating on the teachers' evaluations of the children's performance, and second-trimester marijuana use was significantly associated with reading comprehension and underachievement,[171] altered executive functions including attentional behavior,[172] and altered neural functioning during visuospatial working memory processing.[173] Several studies have reported that *in utero* exposure to cannabis is associated and with ASD[174] and with ADHD.[175]

[168] https://nida.nih.gov/publications/research-reports/marijuana/how-does-marijuana-produce-its-effects.

[169] Campolongo et al. Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. Psychopharmacology (Berl). 2011 Mar;214(1):5-15. doi: 10.1007/s00213-010-1892-x. Epub 2010 Jun 17. PMID: 20556598; PMCID: PMC3045519.

[170] Goldschmidt et al. Effects of prenatal marijuana exposure on child behavior problems at age 10. Neurotoxicol Teratol. 2000 May-Jun;22(3):325-36. doi: 10.1016/s0892-0362(00)00066-0. PMID: 10840176.

[171] Goldschmidt et al. Prenatal marijuana and alcohol exposure and academic achievement at age 10. Neurotoxicol Teratol. 2004 Jul-Aug;26(4):521-32. doi: 10.1016/j.ntt.2004.04.003. PMID: 15203174.

[172] Fried and Smith. A literature review of the consequences of prenatal marihuana exposure. An emerging theme of a deficiency in aspects of executive function. Neurotoxicol Teratol. 2001 Jan-Feb;23(1):1-11. doi: 10.1016/s0892-0362(00)00119-7. PMID: 11274871.

[173] Smith et al. Effects of prenatal marijuana on visuospatial working memory: an fMRI study in young adults. Neurotoxicol Teratol. 2006 Mar-Apr;28(2):286-95. doi: 10.1016/j.ntt.2005.12.008. Epub 2006 Feb 13. PMID: 16473495.

[174] Corsi et al. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. Nat Med. 2020 Oct;26(10):1536-1540. doi: 10.1038/s41591-020-1002-5. Epub 2020 Aug 10. PMID: 32778828; DiGuiseppi et al. Peri-Pregnancy Cannabis Use and Autism Spectrum Disorder in the Offspring: Findings from the Study to Explore Early Development. J Autism Dev Disord. 2022 Nov;52(11):5064-5071. doi: 10.1007/s10803-021-05339-4. Epub 2021 Nov 12. PMID: 34767135; PMCID: PMC9112286; Nutor et al. Prenatal Cannabis Use and Offspring Autism-Related Behaviors: Examining Maternal Stress as a Moderator in a Black American Cohort. J Autism Dev Disord. 2023 Apr 25:1–13. doi: 10.1007/s10803-023-05982-z. Epub ahead of print. PMID: 37097527; PMCID: PMC10127191.

[175] Corsi et al. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. Nat Med. 2020 Oct;26(10):1536-1540. doi: 10.1038/s41591-020-1002-5. Epub 2020 Aug 10. PMID: 32778828; Smith A, Kaufman F, Sandy MS, Cardenas A. Cannabis Exposure During Critical Windows of Development: Epigenetic and Molecular Pathways Implicated in Neuropsychiatric Disease. Curr Environ Health Rep. 2020 Sep;7(3):325-342. doi: 10.1007/s40572-020-00275-4. PMID: 32441004; PMCID: PMC7458902; McLemore and Richardson. Data from three prospective longitudinal human cohorts of prenatal marijuana exposure and offspring outcomes from the fetal period through young adulthood. Data Brief. 2016 Oct 18;9:753-757. doi: 10.1016/j.dib.2016.10.005. PMID: 27833935; PMCID: PMC5096595; Cioffredi LA, Anderson H, Loso H, East J, Nguyen P, Garavan H, Potter A. Prenatal cannabis exposure predicts attention problems, without changes on fMRI in adolescents. Neurotoxicol Teratol. 2022 May-Jun;91:107089. doi: 10.1016/j.ntt.2022.107089. Epub 2022 Mar 18. PMID: 35314358; PMCID: PMC9136933; Tchuente et al. Is in-utero exposure to cannabis associated with the risk of attention deficit with or without hyperactivity disorder? A cohort study within the Quebec Pregnancy Cohort. BMJ Open. 2022 Aug 8;12(8):e052220. doi: 10.1136/bmjopen-2021-052220. PMID: 35940828; PMCID: PMC9364390.


***Figure 16. Marijuana and Pregnancy.*** The American College of Obstetricians and Gynecologists warns that using marijuana during pregnancy may affect the fetus by "Disruption of brain development before birth."[176] Marijuana/cannabis contain Δ9THC, which impacts the same pathway as AM404, an APAP metabolite.

**APAP disruption of prostaglandins during brain development.**

As discussed above, APAP inhibits synthesis of prostaglandins.[177] The impact of APAP on prostaglandins, which mediate the generation of fevers, has been proposed as one of the mechanisms for the anti-pyretic action of APAP.[178] The inhibition of prostaglandins in an adult can be beneficial to the extent it modulates fever or pain, as reviewed above. Unfortunately, inhibiting prostaglandins during brain development disrupts multiple aspects of neural development that are prostaglandin dependent. The prostaglandin E2 is used in neurodevelopment to signal the formation of dendritic spines (the branch-like structures on the

[176] https://www.acog.org/womens-health/infographics/marijuana-and-pregnancy
[177] Flower and Vane. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature (1972) Dec 15;240(5381):410-1. doi: 10.1038/240410a0. PMID: 4564318.; Mitchell et al. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase (1993) Proc Natl Acad Sci USA; 90(24):11693-7. DOI:10.1073/pnas.90.24.11693. PMID: 8265610; PMCID: PMC48050.
[178] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

cell body of a neuron, (Figure 14).[179] In addition, prostaglandins are necessary during brain development, influencing both the arborization and pruning of immature or inappropriate excitatory connections. Exposing rats to APAP near the peak of the brain growth spurt (BGS) at doses clinically relevant to humans was shown to disrupt prostaglandin E2 synthesis and the development of Purkinje neuron cells in the cerebellum.[180] This exposure initially increased dendritic growth, followed by cerebellar atrophy, which then resulted in altered social interactions.

**APAP impacts on vanilloid and serotonergic pathways**

The APAP metabolite AM404 is a "potent activator of vanilloid subtype 1 receptors (TRPV1)."[181] In the brain, TRPV1 receptors are found on neurons, as well as in microglia and astrocytes. Microglia are cells that form the main active immune system for the central nervous system.[182] Astrocytes are a type of glial cell that perform numerous functions, including providing nutrients to neural cells, maintaining ion balance, regulating cerebral blood flow, and maintaining the brain-blood barrier. One function of TRPV1 signaling is to activate inflammatory responses in the brain, and dysfunction of TRPV1 is involved in the development of immune-mediated neurological disorders in the central nervous system.[183] The inflammatory oxidative stress from TRPV1 activation may also contribute synergistically to the oxidative stress caused by the APAP metabolite NAPQI.

The transient receptor potential vanilloid 1 (TRPV1) ion channel is also reported to function in endocannabinoid signaling in microglia, the innate immune cells of the central nervous system (CNS).[184] TRPV1 is a ligand-gated and nonselective cationic channel that is activated by capsaicin (the "heat" molecule in some peppers) and actual heat.[185] In the last few years, a number of studies have suggested a physiological role for AEA as a TRPV1 agonist.[186] The interaction of AEA with TRPV1 and/or CB1 mediates cell death of dopaminergic neurons via cytosolic binding, triggering activation of nonselective ion channels, activation of protein kinases, and increases in intracellular Ca++ concentration, and can result in mitochondrial uncoupling and cell death.[187]

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[179] Tamiji J, Crawford DA. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377

[180] Dean et al. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur J Neurosci. 2012 Apr;35(8):1218-29. doi: 10.1111/j.1460-9568.2012.08032.x. PMID: 22512254; PMCID: PMC3534986.

[181] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

[182] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

[183] Kong, W., Peng Y., Peng B. (2017), Modulation of neuroinflammation: Role and therapeutic potential of TRPV1 in the neuro-immune axis, Brain, Behavior, and Immunity, 64:354-366, DOI: 10.1016/ j.bbi.2017.03.007.

[184] Marinelli Set al. Endocannabinoid signaling in microglia. Glia. 2023 Jan;71(1):71-90. doi: 10.1002/glia.24281. Epub 2022 Oct 12. PMID: 36222019.

[185] Szallasi and Blumberg. Vanilloid (Capsaicin) receptors and mechanisms. Pharmacol Rev. 1999 Jun;51(2):159-212. PMID: 10353985.

[186] De Petrocellis et al. The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. J Biol Chem. 2001 Apr 20;276(16):12856-63. doi: 10.1074/jbc.M008555200. Epub 2001 Jan 26. PMID: 11278420.

[187] Kim et al. Transient receptor potential vanilloid subtype 1 mediates cell death of mesencephalic dopaminergic neurons in vivo and in vitro. J Neurosci. 2005 Jan 19;25(3):662-71. doi: 10.1523/JNEUROSCI.4166-04.2005. PMID: 15659603; PMCID: PMC6725326.

APAP also has indirect effects on serotonergic levels and signaling.[188,189,190] Serotonin signaling facilitates neurogenesis, cell migration, synaptogenesis, and synaptic plasticity.[191] In addition, serotonin activation causes vasoconstriction, which reduces blood flow and restricts the exchange of oxygen, nutrients, and waste products.

**APAP impacts neural crest cell arborization**

Altered structure and communication pathways that occur during early development are hallmarks of ADHD and ASD, as reported in functional and imaging studies of animals and humans (described below). A 2022 study reported on the effects of APAP in altering neuron structures and pathways.[192] The researchers exposed human neurons (NT2N) and chicken neurons (CGN) to APAP at various concentrations and for various durations. Chicken neurons were used in addition to human cells to test the robustness of the results. If the same cause and effect are replicated in cells from different species, it is unlikely that the observed effects were an artifact of the particular human cells used, and more likely that the effects reflect chemical and cellular mechanisms common to all neurons. APAP was reported to disrupt the cytoskeletal proteins SPTBN1 (Figure 17) and TUBB3 in neurons in both human (Figure 17, bottom) and avian (Figure 17, top) cell culture. The left column depicts the controls (Ctrl) and provide a reference for normal *in vitro* growth of structural proteins in neurons that are not exposed to APAP. The next two columns depict the results of neuronal cells exposed to APAP. There is evidence of decreased structural proteins and loss of entire neuronal cells and networks with increasing APAP exposure.

Quantitative analysis of these experiments indicates that APAP disrupted neuronal arborization or branching (Figure 18). Neurons exposed to APAP had a reduction in the number of branch points and a reduction in the length of neurites. The analogy is trees (neurons) with smaller and fewer branches. The effect increased with exposure to higher concentrations of APAP and with exposure to APAP for longer durations.

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[188] Karandikar et al. Effect of drugs modulating serotonergic system on the analgesic action of paracetamol in mice. Indian J Pharmacol. 2016 May-Jun;48(3):281-5. doi: 10.4103/0253-7613.182874. PMID: 27298498; PMCID: PMC4900001.

[189] Pelissier et al. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine3 receptors: in vivo and in vitro evidence. J Pharmacol Exp Ther. 1996 Jul;278(1):8-14. PMID: 8764329.

[190] Dogrul et al. Systemic paracetamol-induced analgesic and antihyperalgesic effects through activation of descending serotonergic pathways involving spinal 5-$HT_7$ receptors. Eur J Pharmacol. 2012 Feb 29;677(1-3):93-101. doi: 10.1016/j.ejphar.2011.12.016. Epub 2011 Dec 21. PMID: 22206817.

[191] Eissa, N., Al-Hougani, M., Sadeq, A., Ojha, SK., Sasses, A., Sadek, B. (2018) Current enlightenment about etiology and pharmacological treatment of autism spectrum disorder, Frontiers in Neurosicence, 12:304, DOI: 10.3389/ fnins.2018.00304, p.2.

[192] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.

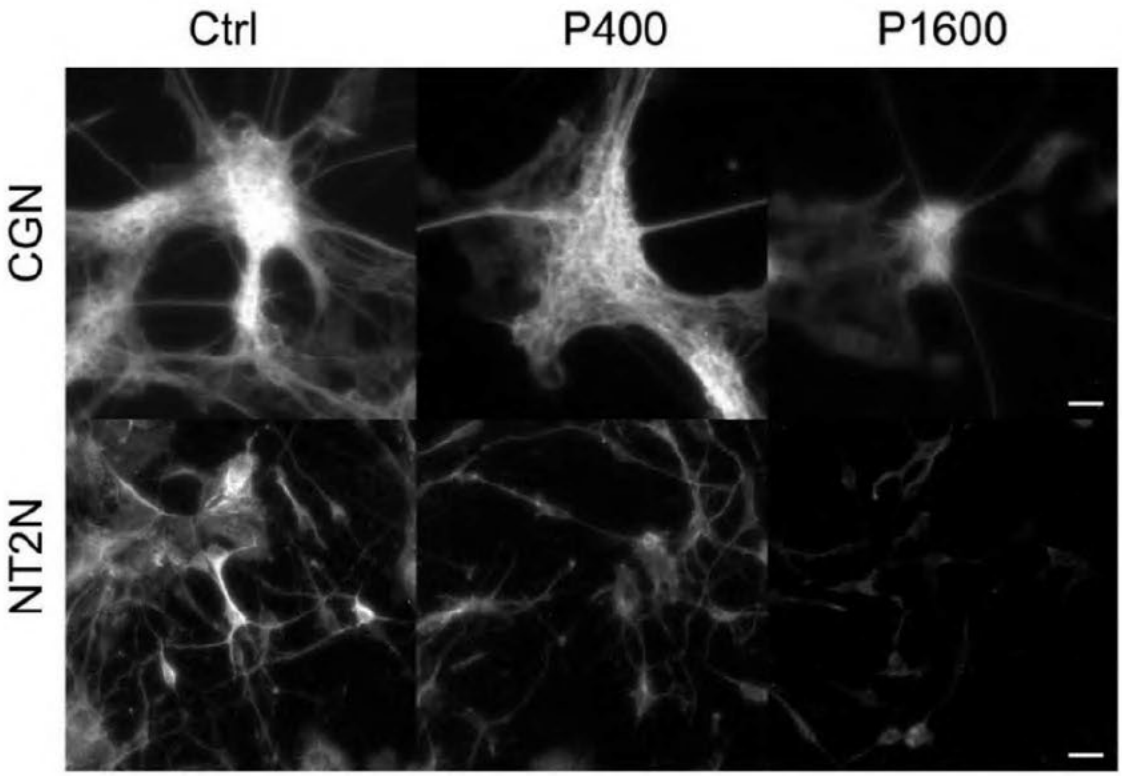


***Figure 17. Changes in Neuronal Structural Protein (Spectrin).*** Fluorescent images of SPTBN1 in control samples of chicken CGNs (top row) and human NT2Ns (bottom row), exposed to increasing concentrations of APAP. Images were taken at 20× magnification, scale bar = 50 µm.[193]

[193] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.

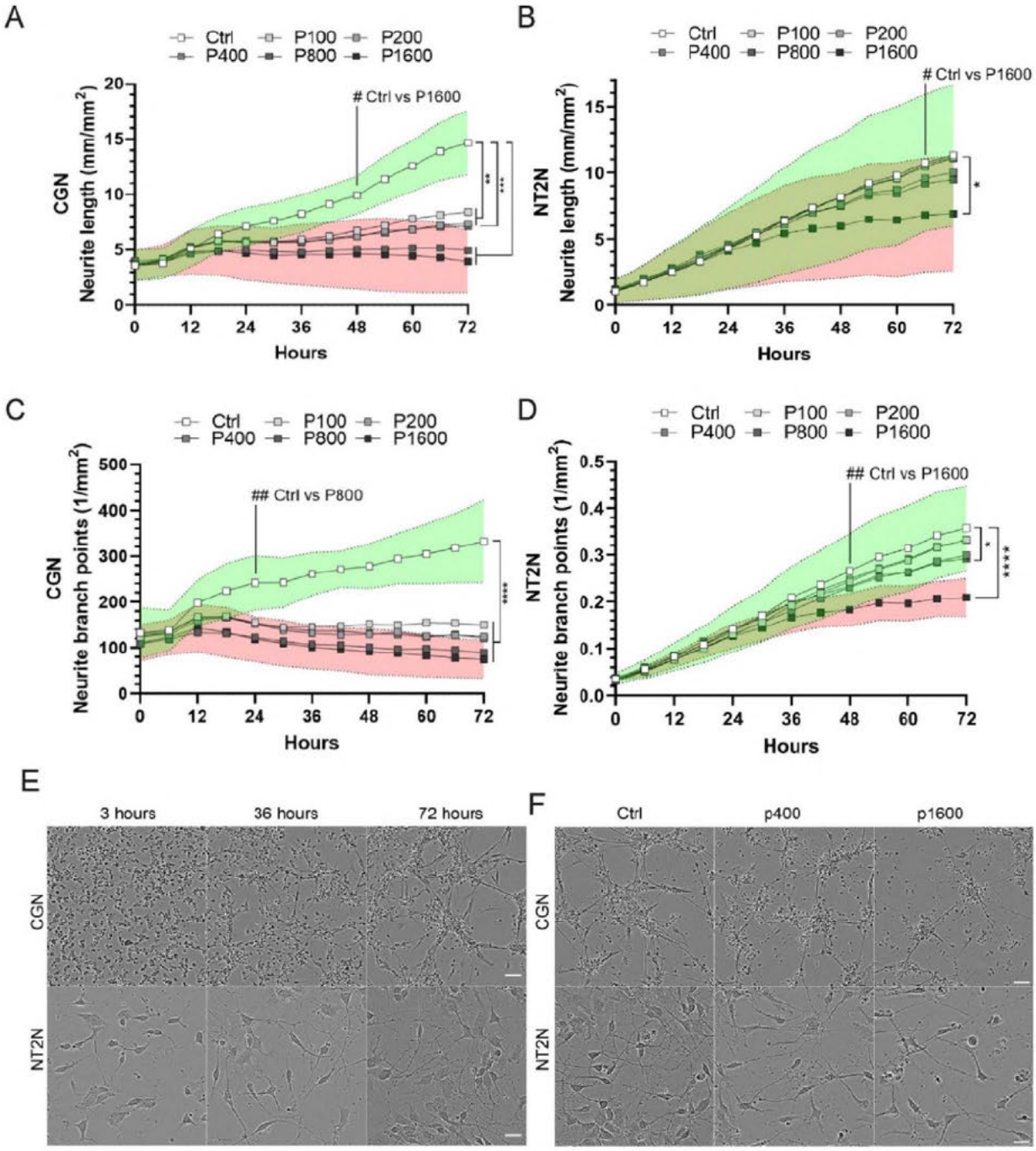


***Figure 18. The Effects of Acetaminophen (APAP) on Neurite Arborization in CGNs and NT2N.*** Cells were exposed to 100–1600 µM APAP for up to 72 h and imaged. The images were used to quantify the neurite lengths of (A) chicken CGNs and (B) human NT2Ns, as well as the neurite branch of points of (C) CGNs and (D) NT2Ns. (E) Representative phase contrast images of CGNs (top row) and NT2Ns (bottom row) from 3 h (first column), 36 h (middle column), and 72 h (last column). (F) Phase contrast images of CGNs (top row) and NT2Ns (bottom row) showing control samples (first column), and samples exposed to APAP at 400 µM (middle column), and 1600 µM (last column). Asterisks denote significance, Dunnett's MCT, $\alpha = 0.05$, ctrl vs sample. Hashes denote first LOAEL, multiples = higher significance. All experiments were performed in biological triplicate, nCGN = 4, nNT2N = 12, 4 images per well. Images were taken at 10× magnification. Data are shown as means, with variance shown as ± SD for controls (green) and P1600 (red).

The Labba study supports that APAP can have harmful effects on the development of the brain and nervous system.[194] Mechanistically, these reported impacts on neurons are consistent with interactions with cannabinoid signaling. Specifically, cannabinoid signaling participates in the modulation of neuronal and glial proliferation. The influence of the APAP metabolite AM404 is also reported to alter endocannabinoid anandamide signaling and can thereby also alter neural cell proliferation. Anandamide also influences embryogenesis, where it is reported to induce a variety of neurological and biological effects, such as the differentiation of neuronal progenitor cells.[195]

**PAP, Anandamide, and Apoptosis in Cortical Neurons**

A study by Schultz et al. investigated the effects of APAP and its metabolite PAP on the viability of mouse-cultured cortical neurons.[196] The authors hypothesized that PAP, which can be metabolized into AM404, see Acetaminophen Metabolism above, and acts as an indirect agonist of cannabinoid receptors, would cause neuronal apoptosis similar to the endocannabinoid anandamide. The test system used in this study was primary cortical neurons prepared from C57BL/6 mouse embryos at embryonic day 15. The control treatments were culture medium with or without ethanol, which was used as a solvent for some of the drugs. The study design was an experimental one, where the neurons were exposed *in vitro* to various concentrations of APAP, PAP, or anandamide for 24 or 48 hours. The statistical methods used were analysis of variance (ANOVA) with Neuman–Keuls post hoc test and two-sample t-test to assess differences between groups. The reported endpoints were cell viability, measured by counting cells labelled with fluorescein diacetate and propidium iodide (live/dead assay) via fluorescent imaging. The conclusions were that PAP and anandamide caused significant loss of neuronal viability at concentrations down to 1 µg/ml, while APAP had no toxic effect at therapeutic doses. The authors suggested that PAP is acting through cannabinoid receptors to induce neuronal apoptosis, and that this has implications for the safety of APAP use in children or individuals with brain injury. According to the authors of the report,

**"We are suggesting that the abnormal brain development seen in autism may be due to the use of acetaminophen at a critical early age."**

As indicated above and in the following sections, the APAP metabolites, including NAPQI, PAP, and AM404, have multiple-related influences on cell behavior and are thereby proposed and reported to disrupt neurodevelopmental cascades via oxidative stress, oxidative damage, and disruption of endocannabinoid signaling.

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[194] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.

[195] Ruiz-Contreras et al. Modulatory Activity of the Endocannabinoid System in the Development and Proliferation of Cells in the CNS. Neurotox Res. 2022 Dec;40(6):1690-1706. doi: 10.1007/s12640-022-00592-6. Epub 2022 Dec 16. PMID: 36522511.

[196] Schultz et al. Effects of the analgesic acetaminophen (Paracetamol) and its para-aminophenol metabolite on viability of mouse-cultured cortical neurons. Basic Clin Pharmacol Toxicol. 2012 Feb;110(2):141-4. doi: 10.1111/j.1742-7843.2011.00767.x. Epub 2011 Aug 20. PMID: 21771276.

### 3. Tissue Interactions

Acetaminophen and key metabolites of APAP reach the developing brain, cause changes consistent with the AOP, and result in ASD and ADHD.

#### i. Increased APAP Concentrations in Fetal Rat Cerebral Spinal Fluid and Brain

Acetaminophen ingested during pregnancy reaches the brain of the fetus. Once APAP is in maternal circulation, it readily passes through the placenta into fetal circulation. For example, in one study 34 pregnant women were given a single dose of APAP upon admission for C-section.[197] Maternal and fetal blood samples were taken at the time of deliver (30 minutes after administering the APAP). The fetal and maternal concentrations of APAP were comparable (11.2μg/mL fetal, 12.3 μg/mL maternal Cmax), which demonstrates that APAP readily crosses the placenta. APAP crosses the placenta and enters fetal blood circulation through the umbilical vein. A unique anatomical feature of the fetal circulatory system called the ductus venosus allows a portion of the highly oxygenated blood from the placenta to bypass the liver and flow directly into the inferior vena cava. From there, the blood is directed towards the right atrium of the fetal heart.[198] This bypass allows APAP to avoid liver metabolism in the fetus, cross the blood brain barrier, and reach the fetal brain.[199] This shunting also limits the ability of the fetal liver to metabolize APAP as it enters fetal circulation, as demonstrated by APAP treatment of Nrf2 mutant mice.[200] Two-thirds of these mutants have congenital intrahepatic shunts, modeling patent ductus venosus, and those mutants with shunts are reported to have diminished hepatoxicity due to APAP exposures (250mg/Kg).

In order to determine APAP exposure levels in the developing brain, Koehn et al. performed a study on rats at three different developmental stages (adults, postnatal day 4 (P4), and gestational day 19 (E19)). They reported **APAP concentrations were two to seven times higher in developing brains compared to adults** (Figure 19, A) and that APAP accumulated in the developing brain in higher amounts than the adult brain. Considering that the adult human therapeutic dose is 1g, and not more than 4g over 24 hours, and the toxic dose is 7g over 24 hours, a seven-fold higher exposure in a developing brain at 2.5hrs is of grave concern regarding embryonic or fetal developmental toxicity. This study also found that chronic treatment of acetaminophen increased this transfer into the fetal brain even more (Figure 19, B), suggesting that the developing brain may be at greater risk from chronic exposure to APAP.

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[197] Nitsche et al. Transplacental Passage of Acetaminophen in Term Pregnancy. Am J Perinatol. 2017 May;34(6):541-543. doi: 10.1055/s-0036-1593845. Epub 2016 Nov 2. PMID: 27806383.

[198] Remien and Majmundar. Physiology, Fetal Circulation. 2023 Apr 26. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan–. PMID: 30969532.

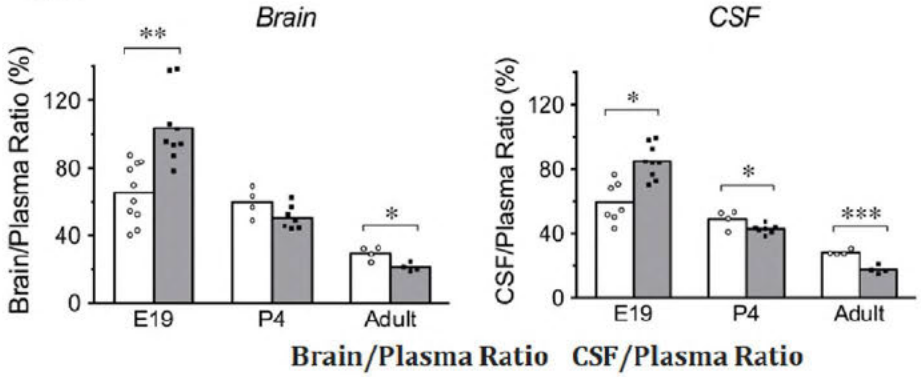
[199] Koehn et al. Determinants of drug entry into the developing brain. F1000Res. 2019 Aug 7;8:1372. doi: 10.12688/f1000research.20078.1. PMID: 31656590; PMCID: PMC6799938.

[200] Skoko et al. Loss of Nrf2 in mice evokes a congenital intrahepatic shunt that alters hepatic oxygen and protein expression gradients and toxicity. Toxicol Sci. 2014 Sep;141(1):112-9. doi: 10.1093/toxsci/kfu109. Epub 2014 Jun 12. PMID: 24924401; PMCID: PMC4271119.

(A) Brain (DPM/µg), CSF (DPM/µl) and Plasma (DPM/µl) Radioactivity Levels

| | | Acute | | | | Chronic | | | |
|---|---|---|---|---|---|---|---|---|---|
| Paracetamol | | n | Brain | CSF | Plasma | n | Brain | CSF | Plasma |
| E19 | Mean | 10 | 39.6 | 35.3 | 57.8 | 9 | 146.1 | 119.0 | 140.5 |
| | SD | (7) | 14.2 | 13.3 | 12.3 | | 32.5 | 16.6 | 15.6 |
| P4 | Mean | 4 | 117.5 | 96.5 | 193.6 | 7 | 84.4 | 71.0 | 166.8 |
| | SD | | 32.4 | 23.1 | 27.6 | | 19.2 | 14.5 | 39.4 |
| Adult | Mean | 4 | 27.3 | 25.4 | 90.1 | 4 | 17.6 | 14.9 | 83.3 |
| | SD | | 20.1 | 15.8 | 58.7 | | 4.2 | 4.5 | 18.5 |

(B) Concentration Ratios




| | Brain/Plasma Ratio | | CSF/Plasma Ratio | |
|---|---|---|---|---|
| Paracetamol | Acute | Chronic | Acute | Chronic |
| E19 | 66 ± 18 | 104 ± 21 | 60 ± 13 | 85 ± 10 |
| P4 | 60 ± 9 | 51 ± 7 | 49 ± 6 | 43 ± 3 |
| Adult | 30 ± 4 | 21 ± 2 | 29 ± 2 | 18 ± 2 |

***Figure 19. Brain, CSF, and Plasma Concentrations for Acetaminophen (APAP).*** APAP concentrations (A, left) at 30 min, (A, right) at 2.5 hours, and (B) the ratio of APAP in brain/plasma at 30minutes (left, white) and 2.5 hours (right, grey). Adults were non-pregnant females and littermates of both sexes were included in the E19 and P4 age groups of pups. SD=standard deviation. * $p<0.05$, ** $p<0.01$, two-tailed Student's t-test. DPM = disintegrations per minute. [201]

### ii. Oxidative Stress and the APAP Metabolites, NAPQI and AM404, in the Brain

The harmful mechanisms described above will reach the brain of the developing fetus. NAPQI itself is generated in brain tissues. APAP easily crosses the blood brain barrier and is distributed uniformly throughout the central nervous system.[202] The liver produces the largest amount of the CYP2E1 enzyme

[201] Koehn et al. Determinants of drug entry into the developing brain. F1000Res. 2019 Aug 7;8:1372. doi: 10.12688/f1000research.20078.1. PMID: 31656590; PMCID: PMC6799938.

[202] Ghanen, C., Perez Mariz, J., Manautou, J., Mottino, A., Acetaminophen; From liver to brain: new insights into drug pharmacological action and toxicity, Pharmacol Res. 2016 July; 109: 119-131, DOI: 10.1016/ j.phrs.2016.02.020, p. 2.

that causes APAP to form the toxic metabolite NAPQI. But the brain also produces CYP2E1 (and other P450 cytochromes), including in the frontal lobe, cerebellum, occipital lobe, and pons regions.[203,204] As a result, APAP present in brain tissue can metabolize into toxic NAPQI. It has been reported that when APAP causes oxidative stress in the brain, it decreases GSH and increases oxidative stress in almost every region of the central nervous system.[205] CYP2E1 is also found in mouse (E17),[206] rat,[207] monkey brain,[208] and prenatal human brain.[209] Epigenetic changes were also reported in CYP2E1 in the placentas from the MARBLES ASD study.[210] This indicates that NAPQI can be produced in all human brain regions examined and in prenatal human brains. CYP2E1 is also found in brain capillaries[211] and is responsive to medications.

The APAP metabolite AM404 also appears in the brain. The enzyme FAAH, which produces the endocannabinoid and serotonergic signaling molecule AM404, shows expression in neurons and throughout the central nervous system.[212] FAAH is expressed in the testis, brain, and other organs (Figure 20). Thus, APAP that reaches the brain can be metabolized into AM404 by FAAH. This gene is also reported to be expressed in human placenta[213] and has been proposed to influence implantation, miscarriage,[214] and placenta cell (trophoblast) proliferation.[215] FAAH is also reported to be expressed in

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[203] Farin and Omiecinski. Regiospecific expression of cytochrome P-450s and microsomal epoxide hydrolase in human brain tissue. J Toxicol Environ Health. 1993 Oct-Nov;40(2-3):317-35. doi: 10.1080/15287399309531797. PMID: 7693960.

[204] Upadhya et al. Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys. 2000 Jan 1;373(1):23-34. doi: 10.1006/abbi.1999.1477. PMID: 10620320.

[205] Micheli et al. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;53:730–6. https://doi.org/10.1016/S0011-393X(05)80745-3

[206] Choudhary et al. Expression patterns of mouse and human CYP orthologs (families 1-4) during development and in different adult tissues. Arch Biochem Biophys. 2005 Apr 1;436(1):50-61. doi: 10.1016/j.abb.2005.02.001. PMID: 15752708.

[207] Joshi and Tyndale. Induction and recovery time course of rat brain CYP2E1 after nicotine treatment. Drug Metab Dispos. 2006 Apr;34(4):647-52. doi: 10.1124/dmd.105.008029. Epub 2006 Jan 24. PMID: 16434548.

[208] Joshi and Tyndale. Regional and cellular distribution of CYP2E1 in monkey brain and its induction by chronic nicotine. Neuropharmacology. 2006 Apr;50(5):568-75. doi: 10.1016/j.neuropharm.2005.11.001. Epub 2005 Dec 20. PMID: 16368115.

[209] Brzezinski et al. Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. J Pharmacol Exp Ther. 1999 Jun;289(3):1648-53. PMID: 10336564.

[210] Zhu et al. Placental DNA methylation levels at CYP2E1 and IRS2 are associated with child outcome in a prospective autism study. Hum Mol Genet. 2019 Aug 15;28(16):2659-2674. doi: 10.1093/hmg/ddz084. PMID: 31009952; PMCID: PMC6687952.

[211] Sato et al. An Atlas of the Quantitative Protein Expression of Anti-Epileptic-Drug Transporters, Metabolizing Enzymes and Tight Junctions at the Blood-Brain Barrier in Epileptic Patients. Pharmaceutics. 2021 Dec 9;13(12):2122. doi: 10.3390/pharmaceutics13122122. PMID: 34959403; PMCID: PMC8708024.

[212] Thomas et al. Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. J Neurosci Res. 1997 Dec 15;50(6):1047-52. doi: 10.1002/(SICI)1097-4547(19971215)50:6<1047::AID-JNR16>3.0.CO;2-1. PMID: 9452020.

[213] Park et al. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta. 2003 Nov;24(10):990-5. doi: 10.1016/s0143-4004(03)00165-6. PMID: 14580383.

[214] Trabucco et al. Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. Placenta. 2009 Jun;30(6):516-22. doi: 10.1016/j.placenta.2009.03.015. Epub 2009 May 5. PMID: 19419760.

[215] Habayeb et al. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology. 2008 Oct;149(10):5052-60. doi: 10.1210/en.2007-1799. Epub 2008 Jul 3. PMID: 18599552.

the pre-implantation mouse embryos[216] and in chicken and mouse embryos before and during neurulation.[217] APAP is also reported to cause a reduction in cerebral cortical fatty acid amide FAAH.[218]

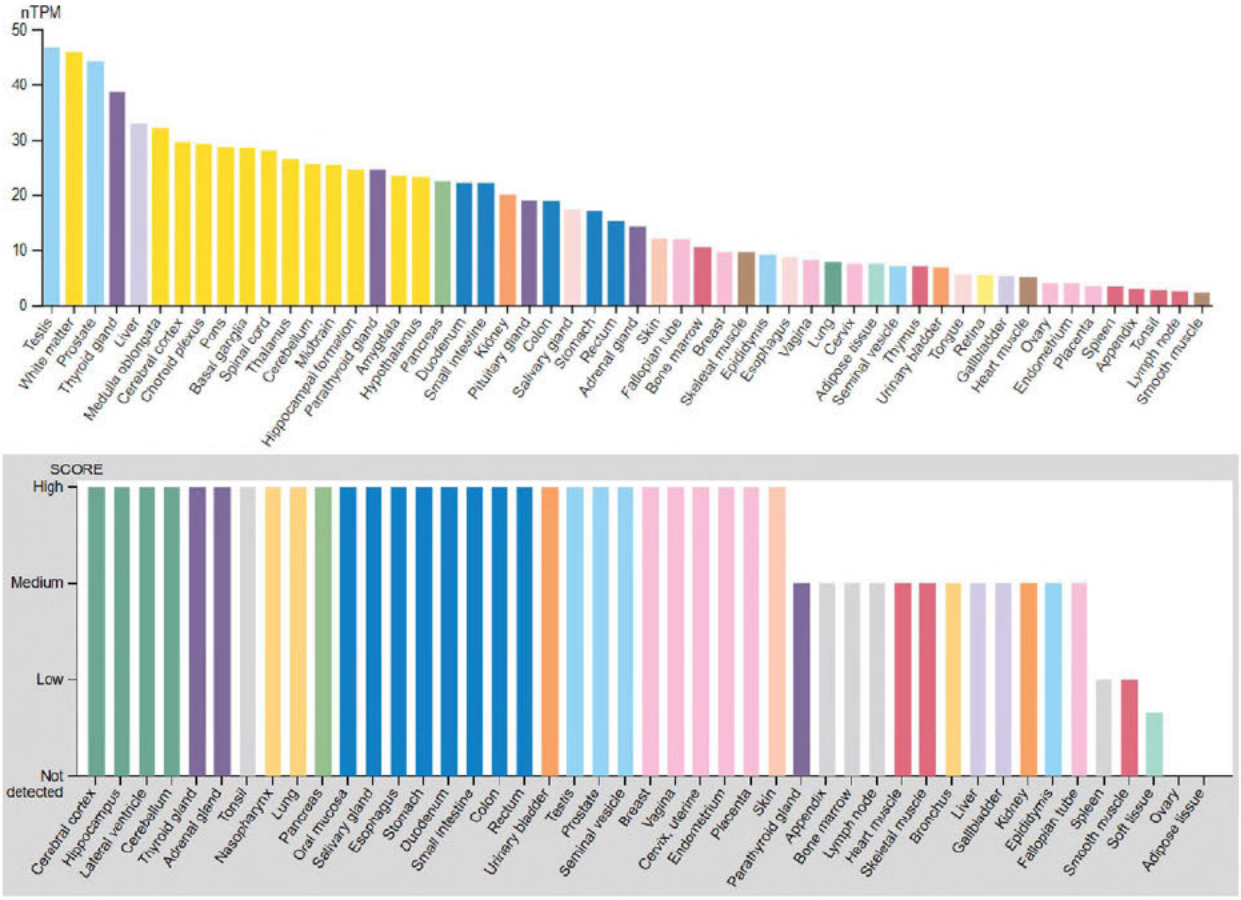


***Figure 20. Gene and Protein Expression of FAAH in Humans.*** (Top) The consensus expression for each gene is shown by organ or tissue as normalized transcripts (nTPM). The expression of FAAH, results in the enzyme that produces AM404 from PAP, with medium transcription (>30 nTPM) in testis, brain, prostate, thyroid, and liver, and low transcription (nTPM > 2) in numerous tissues. (Bottom) Protein expression data is shown for each of the 44 tissues. Color-coding is based on 13 tissue groups, each consisting of tissues with common functional features. Level of antibody staining/expression are indicated for each tissue and are high in neural tissues.[219]

### iii. Oxidative Stress and the Developing Brain

Studies in humans have demonstrated that oxidative stress is produced at "therapeutic" dosages of acetaminophen. In one study, human subjects were given dosages of .5g, 2g, or 4g spread out in four doses

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[216] Paria et al. Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. Biol Reprod. 1999 May;60(5):1151-7. doi: 10.1095/biolreprod60.5.1151. PMID: 10208977.

[217] Psychoyos et al. Cannabinoid receptor 1 signaling in embryo neurodevelopment. Birth Defects Res B Dev Reprod Toxicol. 2012 Apr;95(2):137-50. doi: 10.1002/bdrb.20348. Epub 2012 Feb 6. PMID: 22311661; PMCID: PMC4175447.

[218] Philippot et al. A Cannabinoid Receptor Type 1 (CB1R) Agonist Enhances the Developmental Neurotoxicity of Acetaminophen (Paracetamol). Toxicol Sci. 2018 Nov 1;166(1):203-212. doi: 10.1093/toxsci/kfy199. PMID: 30165669.

[219] Uhlén et al. Proteomics. Tissue-based map of the human proteome. Science. 2015 Jan 23;347(6220):1260419. doi: 10.1126/science.1260419. PMID: 25613900.

over 24 hours (Figure 21).[220] The 4g dosage represents the maximum recommended human dosage over 24 hours. All of these dosages are below dosages reported to result in liver toxicity (≥ 150 mg/kg or 7.5 g in adults, see Acetaminophen Toxicity). Urine and blood were sampled and genomic and metabolomic responses analyzed. The results showed that even at these "therapeutic" dosages, acetaminophen caused oxidative stress, cell death, and DNA damage.

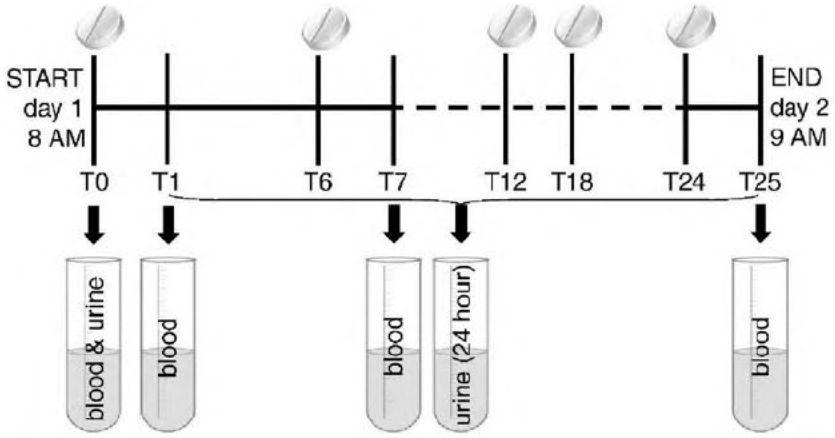


***Figure 21. Sampling and Dosing Schedule.*** Pills indicate APAP dosing time points and tubes indicate sampling of blood and/or urine at the corresponding time point (T in hours) for each dose round. In the 4g APAP dose round, blood samples for transcriptomic analyses were not collected on T1 and T7.[221]

Following the administration of 4 grams of acetaminophen (APAP) at 25 hours (T25), there were reported signals of "apoptosis and survival/oxidative stress/DNA damage". Notably, the process most significantly affected at the 4g dose was oxidative phosphorylation, which was downregulated. Liver toxicity resulting from high and toxic doses of APAP is generally attributed to oxidative stress and impaired mitochondrial function caused by reactive metabolites, such as NAPQI, generated during APAP metabolism. This downregulation of genes involved in oxidative phosphorylation was also observed in previous studies in humans and rats.[222,223] These data support that "therapeutic" doses of APAP induce changes in gene expression consistent with an oxidative stress response. A previous clinical study used a single bolus dose of 4 grams of APAP and also reported gene expression downregulation of oxidative phosphorylation genes.[224] The authors of that study concluded that these changes resembled the gene expression patterns observed in patients who overdosed on APAP and rats receiving toxic doses. These findings indicate that even "therapeutic" doses of APAP produce oxidative stress responses in humans and animals, but this

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[220] Jetten et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol. 2012 Mar 15;259(3):320-8. doi: 10.1016/j.taap.2012.01.009. Epub 2012 Jan 20. PMID: 22285215.

[221] Jetten et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol. 2012 Mar 15;259(3):320-8. doi: 10.1016/j.taap.2012.01.009. Epub 2012 Jan 20. PMID: 22285215.

[222] Fannin et al. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. Hepatology. 2010 Jan;51(1):227-36. doi: 10.1002/hep.23330. PMID: 19918972; PMCID: PMC2925683.

[223] Katyare and Satav. Impaired mitochondrial oxidative energy metabolism following paracetamol-induced hepatotoxicity in the rat. Br J Pharmacol. 1989 Jan;96(1):51-8. doi: 10.1111/j.1476-5381.1989.tb11783.x. PMID: 2522334; PMCID: PMC1854327.

[224] Fannin et al. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. Hepatology. 2010 Jan;51(1):227-36. doi: 10.1002/hep.23330. PMID: 19918972; PMCID: PMC2925683.

stress is recoverable and does not normally result in acute liver disease or severe liver injury. The authors also reasonably suggest that the liver is a resilient and regenerative organ with a large capacity for handling insults in healthy individuals, offering that the liver manages "therapeutic dose" oxidative insults without significant complications for the individual. Unfortunately, the same cannot be said for embryonic or neurodevelopmental tissues. Oxidative stress is not merely something to recover from during development, because oxidative stress alters developmental cascades of cell proliferation, differentiation, and at high levels can produce apoptosis or cell death (Figure 22).

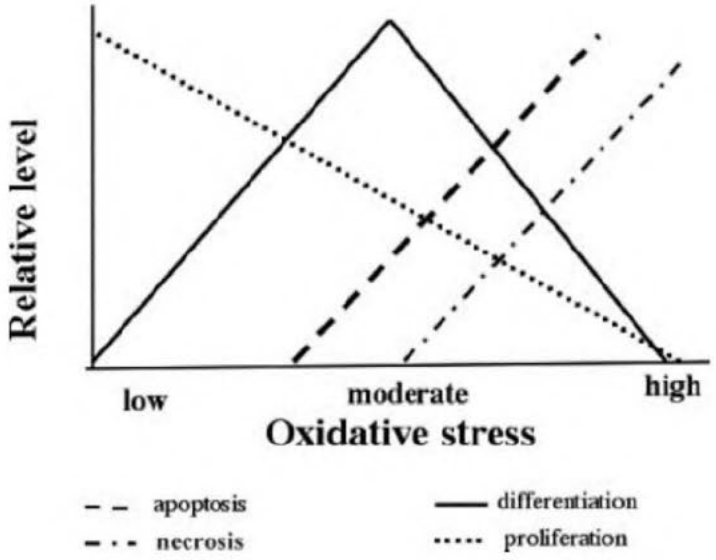


***Figure 22. Effect of Reducing and Oxidative Environments on Cell Proliferation, Differentiation, Apoptosis, and Necrosis.*** In an environment with low oxidative stress, cells are more likely to proliferate (dotted line), whereas a moderately elevated oxidative stress will encourage differentiation (solid line). Higher oxidative stress promotes apoptosis (dashed line) and the highest oxidative environment will promote necrosis (dotted-dashed line).[225]

Multiple studies have examined the impacts of APAP on mitochondria, and these studies indicate that APAP is an inhibitor of complex III in the mitochondrial electron transport chain.[226] While these impacts (Figure 23) were initially reported at hepatotoxic dosages, a recent study reports APAP induced "loss of mitochondrial respiration was also detected below the threshold of acetaminophen toxicity."[227]

[225] Reviewed in Dennery. Effects of oxidative stress on embryonic development. Birth Defects Res C Embryo Today. 2007 Sep;81(3):155-62. doi: 10.1002/bdrc.20098. PMID: 17963268.

[226] Donnelly et al. Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. Arch Toxicol. 1994;68(2):110-8. doi: 10.1007/s002040050043. PMID: 8179480; Meyers et al. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol Appl Pharmacol. 1988 May;93(3):378-87. doi: 10.1016/0041-008x(88)90040-3. PMID: 3368917.

[227] Prill et al. Real-time monitoring of oxygen uptake in hepatic bioreactor shows CYP450-independent mitochondrial toxicity of acetaminophen and amiodarone. Arch Toxicol. 2016 May;90(5):1181-91. doi: 10.1007/s00204-015-1537-2. Epub 2015 Jun 4. PMID: 26041127.

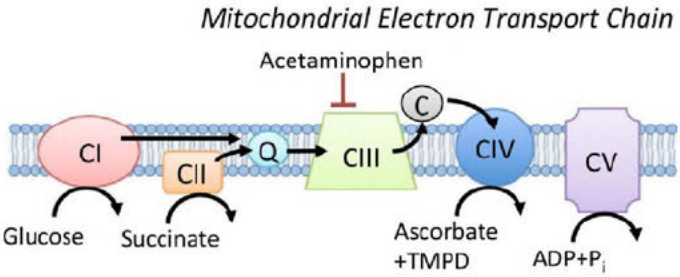


***Figure 23. Acetaminophen Directly Inhibits Mitochondrial Complex III Independent of CYP450.***

There are similar reports in the brain, such that NAPQI "exerts oxidative stress and depletes glutathione in the brain" even at relatively low doses, below the level that results in liver toxicity.[228] This study also reported that the developing brain has lower levels of GSH than the adult brain, which makes the developing brain less resistant to oxidative stress, and more likely to have adverse impacts by oxidative stress on the developing brain. The presence of "double bonds in membrane phospholipids makes them particularly susceptible to oxidative damage" so the brain is particularly vulnerable to oxidative stress because it has high lipid content and limited antioxidant capacity.[229] As indicated previously, oxidative stress and ROS can decrease GSH, and induce DNA oxidation, and lipid peroxidation, which can disrupt the composition of membrane phospholipids and alter neuronal function.

Furthermore, some individuals and populations are reported to have differences in glutathione and APAP metabolism, which can influence the generation of and increase vulnerability to oxidative stress during neurodevelopment.[230] For example, African-Americans oxidize acetaminophen more slowly than European-Americans, which is partially explained by reported CYP2E1 polymorphisms.[231] The resultant increased exposure to NAPQI is expected to increase risk of lowering GSH, increased DNA or lipid oxidation, protein adducts, and neurodevelopmental disorders. As expected, studies have shown that children with autism often have lower levels of GSH, and increased levels of oxidized glutathione (GSSG).[232]

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[228] Bührer et al. Paracetamol (Acetaminophen) and the Developing Brain. Int J Mol Sci. 2021 Oct 15;22(20):11156. doi: 10.3390/ijms222011156. PMID: 34681816; PMCID: PMC8540524.

[229] Tamiji and Crawford. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377.

[230] Upadhya et al. Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys. 2000 Jan 1;373(1):23-34. doi: 10.1006/abbi.1999.1477. PMID: 10620320.

[231] Court MH, Zhu Z, Masse G, Duan SX, James LP, Harmatz JS, Greenblatt DJ. Race, Gender, and Genetic Polymorphism Contribute to Variability in Acetaminophen Pharmacokinetics, Metabolism, and Protein-Adduct Concentrations in Healthy African-American and European-American Volunteers. J Pharmacol Exp Ther. 2017 Sep;362(3):431-440. doi: 10.1124/jpet.117.242107. Epub 2017 Jun 29. PMID: 28663312; PMCID: PMC5562097.

[232] James et al. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. Am J Clin Nutr. 2004 Dec;80(6):1611-7. doi: 10.1093/ajcn/80.6.1611. PMID: 15585776; Geier et al. A prospective study of transsulfuration biomarkers in autistic disorders. Neurochem Res. 2009 Feb;34(2):386-93. doi: 10.1007/s11064-008-9782-x. Epub 2008 Jul 9. Erratum in: Neurochem Res. 2009 Feb;34(2):394. PMID: 18612812.